Polyphosphate modulates blood coagulation and fibrinolysis

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Inorganic polyphosphate is an abundant component of acidocalcisomes of bacteria and unicellular eukaryotes. Human platelet dense granules strongly resemble acidocalcisomes, and we recently showed that they contain substantial amounts of polyphosphate, which is secreted upon platelet activation. We now report that polyphosphate is a potent hemostatic regulator, accelerating blood clotting by activating the contact pathway and promoting the activation of factor V, which in turn results in abrogation of the function of the natural anticoagulant protein, tissue factor pathway inhibitor. Polyphosphate was also found to delay clot lysis by enhancing a natural antifibrinolytic agent, thrombin-activatable fibrinolysis inhibitor. Polyphosphate is unstable in blood or plasma, owing to the presence of phosphatases. We propose that polyphosphate released from platelets or microorganisms initially promotes clot formation and stability; subsequent degradation of polyphosphate by blood phosphatases fosters inhibition of clotting and activation of fibrinolysis during wound healing.

factor V | platelets | tissue factor pathway inhibitor | acidocalcisomes | dense granules

Polyphosphate (polyP) is widely distributed in biology, being found in bacteria, fungi, plants, and animals (1). The biologic functions of polyP have been studied most extensively in prokaryotes and unicellular eukaryotes, in which high levels of polyP accumulate in acidic organelles known as acidocalcisomes. PolyP in these organisms can reach chain lengths of several hundred phosphate units. In unicellular organisms, polyP has been shown to play essential roles in stress responses and virulence (1, 2), although its function in higher eukaryotes, including man, has not been extensively investigated. Recently, we reported that dense granules of human platelets strongly resemble acidocalcisomes and contain millimolar levels of polyP (with chain lengths of ≈70–75 phosphate units), making acidocalcisomes the only known class of organelle conserved during evolution from bacteria to humans (3). Human platelets each have three to eight dense granules (also called δ granules), a type of secretory granule that contains serotonin, ADP, ATP, and PP_i in addition to polyP. Patients with dense granule defects exhibit bleeding diatheses, underscoring the role of these secretory granules in hemostasis (4). Platelets contain ≈0.74 nmol polyP per 10^8 platelets, which is secreted after stimulation by platelet agonists such as thrombin (3). Therefore, released polyP could readily attain a concentration of 3 μM in whole blood, and this could be far higher in platelet-rich thrombi. (Concentrations of polyP are expressed in this paper as phosphate monomer.)

The importance of platelets in hemostasis suggested to us that polyP may play an important role in the blood clotting system. In this study, we found that polyP of the size released from activated platelets has a strongly net procoagulant effect, which is exerted at several levels in the clotting/fibrinolysis system: PolyP activates the contact or intrinsic pathway of blood clotting, accelerates the activation of coagulation factor V by both thrombin and factor Xa, abrogates the function of an important natural anticoagulant protein, tissue factor pathway inhibitor (TFPI), and enhances the action of a natural inhibitor of fibrinolysis, thrombin-activatable fibrinolysis inhibitor (TAFI).

Results and Discussion

PolyP Accelerates Clotting. We tested the idea that polyP could modulate blood coagulation by using an *in vitro* model of clotting and fibrinolysis in which clotting is induced by adding excess Ca^{2+} to citrated human plasma and fibrinolysis is stimulated by urokinase-type plasminogen activator (uPA). (All of the clotting tests in this study used platelet-poor plasma.) Fig. 1*A* shows that, in the absence of polyP, plasma clotted slowly (25 min) and the clot lysed thereafter (50% lysis by 43 min). Adding polyP of chain length 75 ($polyP_{75}$) accelerated clotting (<5 min) and greatly delayed fibrinolysis (50% lysis at 68 min). Shortening of the clot time suggested that polyP can trigger the clotting cascade, most likely through the contact (intrinsic) pathway. Indeed, polyP shortened the clotting time when preincubated for 2 or 3 min with normal plasma before adding Ca^{2+}, and order-of-addition experiments using mixtures of normal and factor XII-deficient plasma demonstrated that polyP can trigger clotting only in the presence of factor XII (Fig. 1*B*), demonstrating that polyP is an activator of the contact pathway of blood clotting. $PolyP_{75}$ also shortened plasma clotting times initiated by tissue factor or factor Xa, but not thrombin (data not shown), indicating that it can affect the clotting cascade at the level of factor Xa and/or factor VIIa in addition to activating the contact pathway. This latter effect could result from antagonizing the function of a plasma coagulation inhibitor. Two important protease inhibitors that target factors VIIa and Xa are antithrombin and TFPI (5). PolyP did not block antithrombin's anticoagulant effect in factor Xa-initiated clotting assays, however (Fig. 6, which is published as supporting information on the PNAS web site).

PolyP Abrogates TFPI Function. The Kunitz-type plasma protease inhibitor, TFPI, targets the active sites of both coagulation factors VIIa and Xa (5). The ability of polyP to interfere with the anticoagulant function of TFPI was examined in tissue factor clotting assays in which varying concentrations of recombinant TFPI were added to plasma. Plasma normally contains relatively low levels of free TFPI (5), so adding exogenous TFPI can dramatically lengthen clotting times (Fig. 2*A*). Strikingly, $polyP_{75}$

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Abbreviations: polyP, polyphosphate; TFPI, tissue factor pathway inhibitor; TAFI, thrombin-activatable fibrinolysis inhibitor; uPA, urokinase-type plasminogen activator; PCPS, 80% phosphatidylcholine/20% phosphatidylserine; CPI, potato carboxypeptidase inhibitor.

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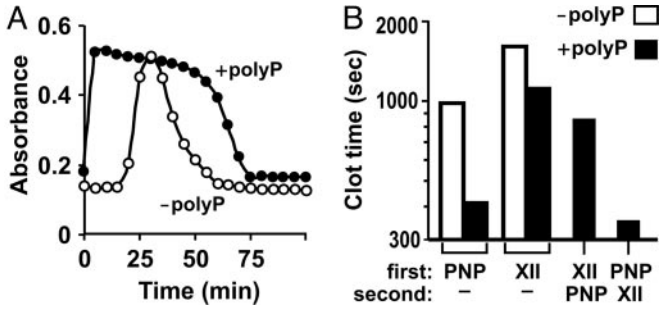

Fig. 1. PolyP activates clotting and delays fibrinolysis. (*A*) Combined clotting/fibrinolysis assays were conducted in 96-well plates by adding 75 μM polyP$_{75}$ 3 min before adding Ca^{2+} and uPA (done in duplicate; representative of ten experiments). In the presence of polyP, plasma clotted faster and the clots lysed more slowly. (*B*) Clotting assays were conducted as in *A* except that no uPA was added and plasma was preincubated for 2 min with 0 or 75 μM polyP$_{75}$ before the addition of Ca^{2+}. The first two bars on the left show that addition of polyP to pooled normal plasma (PNP) dramatically shortened the clotting time, whereas the next two bars show that addition of polyP to factor XII-deficient plasma (XII) did not. The two bars on the right are the results of, first, preincubating 40 μl plasma (factor XII-deficient or normal) with 75 μM polyP$_{75}$ for 2 min, and second, adding an additional 40-μl aliquot of normal or factor XII-deficient plasma, respectively, followed immediately by the addition of Ca^{2+} to initiate clotting. PolyP dramatically shortened the clotting time only when preincubated with normal plasma, not with factor XII-deficient plasma, even though the final plasma mixtures were identical.

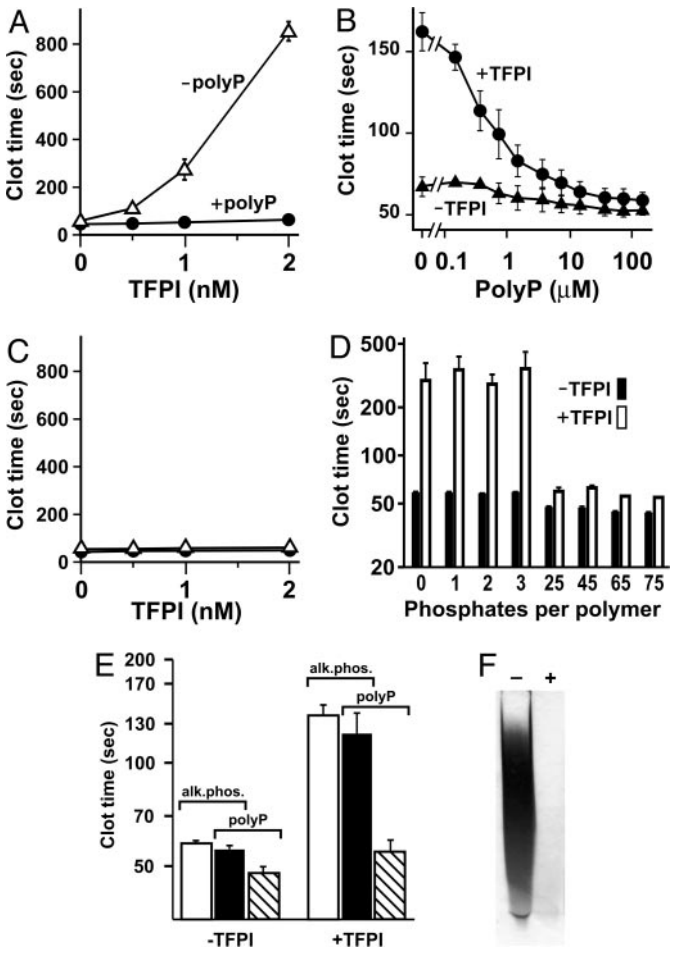

Fig. 2. PolyP abrogates the anticoagulant effect of TFPI. (*A*) Tissue factor-induced clotting times were measured in a coagulometer using normal plasma and the indicated concentrations of recombinant TFPI, in the presence or absence of 25 μM polyP$_{75}$. (*B*) Concentration dependence of the abrogation of TFPI's anticoagulant effect by polyP. Clotting assays were performed with normal plasma as in *A* containing 0 or 1 nM recombinant TFPI and varying concentrations of polyP$_{75}$. Data are mean clotting times ± SEM. (*C*) Factor Va abrogates TFPI function. Clotting assays were performed as in panel *A* using factor V-deficient plasma supplemented with 1 nM factor Va in the presence (filled circles) or absence (open triangles) of 25 μM polyP$_{75}$. (*D*) The ability of polyP to abrogate TFPI function depends on chain length. Clotting assays were performed as in panel *B* with 0 or 2 nM TFPI, and with 25 μM polyP of the indicated chain lengths. Mean clotting times (±SEM) are depicted. (*E*) Phosphatase digestion of polyP eliminated its ability to abrogate TFPI function. PolyP$_{65}$ (7.5 mM) was digested for 2 h at room temperature with 0 or 100 units/ml calf intestinal alkaline phosphatase, then diluted for use in clotting assays. Clotting assays were performed as in *B* with 0 or 1 nM TFPI, and mixed with a dilution of the phosphatase reaction mixtures to yield a final concentration of 4.3 μM polyP$_{65}$. Data are mean clotting times ± SEM. (*F*) Urea-PAGE analysis of polyP$_{65}$ after 2 h incubation with (+) or without (−) alkaline phosphatase; polyP was stained with toluidine blue.

completely abrogated the anticoagulant effect of TFPI at all TFPI concentrations tested. A similar reversal of TFPI's anticoagulant effect was seen when clotting was initiated with factor Xa (Fig. 7, which is published as supporting information on the PNAS web site). The ability of polyP$_{75}$ to block TFPI's anticoagulant effect was concentration dependent (Fig. 2*B*): As little as 375 nM polyP appreciably shortened the TFPI-prolonged clotting time, whereas 5 μM polyP$_{75}$ completely neutralized TFPI's anticoagulant effect. The ability of polyP to abrogate TFPI function depended on polyP chain length, as polyP$_{25}$, polyP$_{45}$, polyP$_{65}$, and polyP$_{75}$ all neutralized the anticoagulant effect of TFPI, whereas P_i, PP_i, or polyP$_3$ did not (Fig. 2*D*). These micromolar polyP concentrations are far below the final Ca^{2+} concentration used in our clotting assays (8.3 mM final), ruling out the possibility that polyP influences clotting simply by reducing the free Ca^{2+} concentration. In addition, 25 μM polyP$_{75}$ still abrogated the anticoagulant effect of TFPI at 10.3 mM Ca^{2+} (not shown). Other charged polymers, included single- and double-stranded DNA, failed to abrogate TFPI function (Fig. 8, which is published as supporting information on the PNAS web site), indicating that other linear, phosphate-containing polymers do not recapitulate the specific effects of polyP.

Extensive digestion of polyP$_{65}$ with calf intestine alkaline phosphatase, which is also a highly active exopolyphosphatase (6), rendered it completely ineffective in blocking the anticoagulant effect of TFPI (Fig. 2*E*), and gel electrophoresis revealed that polyP was completely degraded (Fig. 2*F*). This finding confirmed that short phosphate polymers cannot block the anticoagulant effect of TFPI. It also showed that the ability of polyP to reverse the anticoagulant effect of TFPI is not due to contaminants present in the polyP preparations.

PolyP Accelerates Factor V Activation and Thrombin Generation. We found that polyP was unable to block the inhibitory function of TFPI in experiments using purified factor Xa or the tissue factor-factor VIIa complex (Fig. 9, which is published as supporting information on the PNAS web site). This finding indicated that other components of plasma mediate polyP's abrogation of TFPI function. Mast and Broze (7) reported that factor Xa, when bound to its protein cofactor, factor Va, is refractory to inhibition by TFPI, especially in the presence of its substrate, prothrombin. We confirmed this: Replacing factor V with factor Va in clotting assays totally abrogates TFPI function (compare Fig. 2 *C* and *A*). Therefore, accelerating the activation of factor V could explain the ability of polyP to block the anticoagulant function of TFPI. Mixing purified factors Xa, V and prothrombin results in thrombin generation once the factor V is converted to Va. (Factor V activation can be catalyzed directly by factor Xa or through back-activation by thrombin.) Including polyP in such

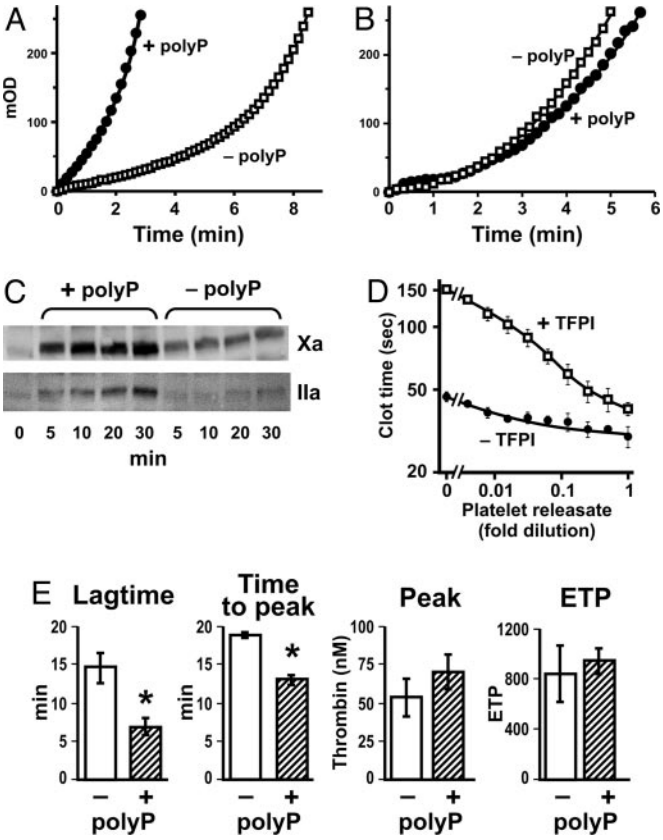

Fig. 3. PolyP accelerates the activation of factor V and the generation of thrombin. (*A*) Activation of prothrombin is accelerated by polyP plus factor V. Reactions contained 6 nM factor V, 0.33 nM factor Xa, and 400 nM prothrombin with or without 22.5 μM $polyP_{75}$. The *y* axis represents cleavage of the thrombin substrate, S-2238. (*B*) Activation of prothrombin is not accelerated by polyP in the presence of factor Va. Reactions were identical to *A* except that 0.45 nM factor Va was used in place of factor V. (*C*) PolyP accelerates factor V activation by factor Xa (Xa) and thrombin (IIa). Factor V (100 nM) was reacted with either 1 nM factor Xa or 0.1 pM thrombin for the indicated times, aliquots were inactivated by heating in SDS sample buffer and resolved on SDS PAGE followed by Western blotting, and the factor Va heavy chain was detected by using a specific monoclonal antibody. (*D*) Platelet releasates abrogate the anticoagulant function of TFPI. Clotting assays were performed as in Fig. 2*A*, containing 0 or 1 nM TFPI and the indicated dilution of platelet releasate (1 = undiluted; 0 = no releasate added) prepared from platelets resuspended at 6.0×10^9 per ml. Data are mean ± SEM ($n = 3$) from a single donor's platelets, but are representative of releasates obtained from five separate donors. (*E*) PolyP shortens the lag to thrombin generation. Plasma clotting was initiated by tissue factor with or without 75 μM $polyP_{75}$, and thrombin generation was monitored in real time by using a fluorogenic substrate for thrombin and analyzed in a Thrombinoscope. Lag time is the time to achieve 10 nM thrombin, whereas ETP is endogenous thrombin potential (integrated thrombin generation over 60 min). Data are mean ± SEM ($n = 4$); asterisks indicate statistical significance ($P < 0.0001$ using Student's *t* test).

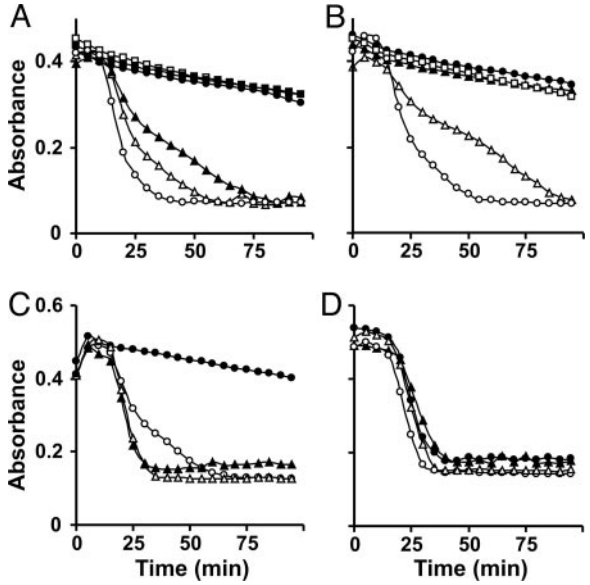

Fig. 4. PolyP inhibits fibrinolysis in a TAFI-dependent fashion. Combined clotting/fibrinolysis assays were performed in 96-well plates as in Fig. 1*A*, except that 10 nM thrombin was added to rapidly initiate clotting. (*A*) PolyP inhibition of fibrinolysis is concentration dependent. Assays were performed with pooled normal plasma in the presence of 0 nM (open circles), 750 nM (open triangles), 3.75 μM (filled triangles), 7.5 μM (open squares), 37.5 μM (filled squares), or 75 μM (filled circles) $polyP_{75}$. (*B*) The antifibrinolytic effect of polyP depends on chain length. PolyP of different chain lengths was included at 75 μM: $PolyP_{25}$ (open triangles), $polyP_{45}$ (filled triangles), $polyP_{65}$ (open squares), $polyP_{75}$ (filled circles), or no polyP (open circles). (*C* and *D*) Attenuation of fibrinolysis by polyP is TAFI dependent. (*C*) Assays with normal plasma were performed in the absence (open circles) or presence of 75 μM $polyP_{75}$ (filled circles). Addition of 6.25 μM CPI accelerated clot lysis equally well in the absence (open triangles) and the presence of $polyP_{75}$ (filled triangles). (*D*) Assays with TAFI-deficient plasma were performed without CPI or polyP (open circles), with 75 μM $polyP_{75}$ but no CPI (filled circles), with 6.25 μM CPI but no polyP (open triangles), or with both 75 μM $polyP_{75}$ and 6.25 μM CPI (filled triangles). Results in all four panels are the mean of duplicate wells (representative of three to five experiments).

a reaction mixture greatly accelerated the initial rate of thrombin generation (Fig. 3*A*). In a parallel experiment in which factor V was replaced with factor Va, polyP did not accelerate thrombin generation at all (Fig. 3*B*), suggesting that polyP acts by promoting factor V activation. Directly examining factor V activation catalyzed by factor Xa or thrombin using purified proteins demonstrated that polyP greatly accelerated factor V activation by both proteases (Fig. 3*C*).

Platelet alpha granules contain factor V, which is secreted upon platelet activation (8). Platelet factor V is more active in supporting clotting than is plasma factor V, and in particular, platelet factor V is activated by factor Xa at an accelerated rate relative to plasma factor V (9). Platelet factor V is structurally modified compared to the plasma protein, which may help explain its enhanced activity (8, 10). We propose that an additional factor enhancing the procoagulant function of platelet factor V is the concomitant secretion of polyP upon platelet activation, and that polyP serves as a cofactor to enhance the rate of factor V activation by both thrombin and factor Xa. Consistent with this notion, platelet releasates abrogated TFPI anticoagulant function in clotting assays (Fig. 3*D*). If polyP accelerates factor V activation during clotting of plasma, it should shorten the time lag before the appearance of thrombin. We examined this in plasma clotting reactions in real time and found that adding polyP significantly shortened the lag time for thrombin generation and also the time to peak thrombin generation (Fig. 3*E*). However, polyP did not alter the total amount of thrombin generated.

PolyP Delays Fibrinolysis. We next turned our attention to understanding how polyP delays fibrinolysis, using assays in which plasma is rapidly clotted with thrombin in the presence of uPA (Fig. 4). In the absence of polyP, the clots subsequently lysed (50% lysis by 20 min), whereas in the presence of $polyP_{75}$, fibrinolysis was inhibited in a concentration-dependent manner (Fig. 4*A*). At $polyP_{75}$ concentrations of 0.75 or 3.8 μM, lysis was delayed to 25 and 40 min, respectively, whereas at 7.5 μM $polyP_{75}$, the time to 50% lysis exceeded 100 min. Similar effects were also observed when tPA was

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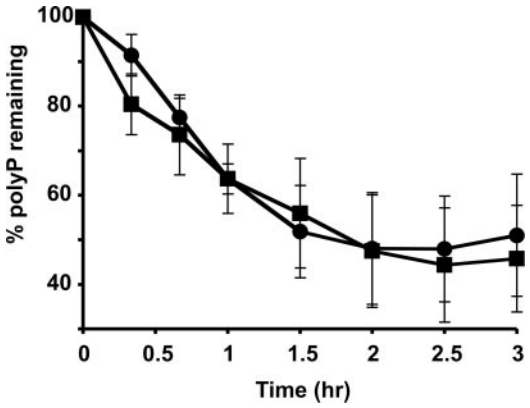

Fig. 5. Instability of polyP in plasma and serum. $PolyP_{75}$ (10 mM) was incubated in human serum (squares) or heparinized plasma (circles) at 37°C. At different time points, aliquots were taken and the remaining polyP was determined after guanine isothiocyanate extraction as described (3).

substituted for uPA in this clot lysis model (data not shown). The polyP chain length was critical: $PolyP_{45}$, $polyP_{65}$, and $polyP_{75}$ inhibited fibrinolysis, whereas $polyP_{25}$ had reduced inhibitory ability (Fig. 4*B*). P_i, PP_i, and $polyP_3$ had no discernable effect on fibrinolysis (not shown).

$PolyP_{75}$ did not inhibit the enzymatic activity of purified uPA or tPA (Fig. 10, which is published as supporting information on the PNAS web site). Therefore, we hypothesized that polyP might enhance the function of one of the inhibitors of fibrinolysis, possibly TAFI (also known as procarboxypeptidase B, U, or R), a plasma procarboxypeptidase that serves as a link between coagulation and fibrinolysis (11). When activated by thrombin, TAFIa decreases the rate of fibrinolysis by removing C-terminal lysine residues from fibrin, which normally bind plasminogen and tPA and accelerate the generation of plasmin (12–14). We first examined this question by using potato carboxypeptidase inhibitor (CPI) (15), a specific inhibitor of TAFIa in plasma. Pooled normal plasma was clotted by adding Ca^{2+} and thrombin in the presence or absence of $polyP_{75}$. Adding CPI to plasma enhanced the rate of fibrinolysis, consistent with elimination of endogenous TAFIa activity (Fig. 4*C*). Interestingly, $polyP_{75}$ was unable to down-regulate fibrinolysis in the presence of CPI, indicating that the antifibrinolytic nature of polyP is mediated through TAFIa. In addition, $polyP_{75}$ had no effect on clot lysis time in TAFI-deficient plasma (Fig. 4*D*). These data strongly indicate that polyP acts through a TAFIa-dependent mechanism to inhibit lysis and confer fibrin stabilization. Studies with purified proteins failed to show any direct effect of polyP on the rate of TAFI activation by either thrombin or plasmin, nor did polyP have any effect on the thermal stability of TAFIa in plasma (Fig. 11, which is published as supporting information on the PNAS web site). Therefore, the ability of polyP to delay fibrinolysis in a TAFIa-dependent fashion likely results from its ability to accelerate thrombin generation as shown above, thereby resulting in earlier formation of TAFIa during the time course of clotting. Earlier activation of TAFI may allow it more time to remove C-terminal lysine residues from newly formed fibrin strands, potentiating its antifibrinolytic effect.

Stability of PolyP in Plasma. The stability of polyP was investigated by adding $polyP_{75}$ to human serum or heparinized plasma and incubating at 37°C. The results (Fig. 5) revealed that $polyP_{75}$ is unstable in this setting, with 50% degradation at ≈2 h. This finding confirms a previous report on the lability of $polyP_{34}$ in human plasma or serum (16).

Role of PolyP in Clotting and Fibrinolysis. Our results show that polyP, an ancient molecule ubiquitous in many microorganisms and abundant in human platelets, is a potent modulator of blood coagulation and fibrinolysis. PolyP acts at two points in the clotting cascade with consequences for both clotting and fibrinolysis: It activates the contact pathway of blood clotting and it accelerates the conversion of factor V to Va. Of the two, accelerating the activation of factor V is probably the more important. Rapid formation of factor Va antagonizes the function of an important natural anticoagulant (TFPI), whereas accelerated thrombin generation likely enhances the function of a natural antifibrinolytic protein (TAFI). Although plasma contains low levels of free TFPI, both TAFI and TFPI are present in human platelets and are secreted following platelet activation (17, 18). In addition, a large pool of active TFPI is sequestered on the endothelial surface *in vivo* (5), so the local concentration of active TFPI in blood clots can be considerably higher than in plasma.

PolyP is secreted after platelet activation (3), likely resulting in the simultaneous release of polyP, factor V, TFPI, and TAFI during the onset of coagulation. Thus, the burst of polyP released from platelet dense granules may enhance coagulation by accelerating the activation of factor V, thereby abrogating the anticoagulant effect of TFPI; and it may protect the clot from premature lysis by enhancing the production of TAFIa through accelerated thrombin generation. PolyP is inherently unstable in plasma, owing to the presence of phosphatases that degrade it over a matter of hours. Very short-chain phosphate molecules were devoid of ability to enhance blood clotting or to delay fibrinolysis. Therefore, polyP released from activated platelets or lysed microorganisms could enhance clot formation and stability immediately after vascular injury. Several hours later, polyP would be neutralized in the clot milieu through the action of plasma phosphatases. At this time, the coagulation inhibitor, TFPI, would become fully active and fibrinolysis would no longer be opposed by polyP. This would help limit the further expansion of the blood clot and would also allow wound healing to occur through the dissolution of fibrin. The bleeding tendencies of patients with dense granule defects support the concept that polyP may play a role in assisting normal hemostasis (4).

PolyP with chain lengths ≥45 phosphate units effectively modulated plasma clotting and fibrinolysis at concentrations that are readily achievable *in vivo* after platelet activation. The concentrations of soluble polyP in plasma and serum have been reported by others to be 12–14 μM in plasma and 39–49 μM in serum (16). The higher values in serum are consistent with release of polyP from cells such as platelets during blood clotting. How much of the endogenous polyP in plasma corresponds to polyP with chain lengths of 45 and greater, versus smaller polyP molecules, requires further study.

In addition to its role in hemostasis, the blood clotting system plays an important role in host defense against pathogens by stimulating inflammation, fibrin deposition, and possibly other mechanisms (19–22). In addition, the contact pathway of blood clotting, although dispensable for normal hemostasis, appears to play important roles in kinin generation and host–pathogen interactions (23). PolyP is abundant in many microorganisms, including certain pathogens, and may be released when these cells lyse. Based on the studies presented here, release of polyP by infectious microorganisms could have potent procoagulant and antifibrinolytic effects, enhancing the host defense role of the blood clotting and fibrinolytic systems. In addition, the ability of polyP released by activated platelets and/or bacteria to severely abrogate TFPI function might limit the effectiveness of TFPI as a drug (Tifacogin) in combating disseminated intravascular coagulation in sepsis (24).

Materials and Methods

Materials. Pooled normal plasma and factor XII-deficient plasma were from George King Bio-Medical (Overland Park, KS); plasma immunodepleted of TAFI or antithrombin was from Affinity Biologicals (Ancaster, Ontario, Canada); and factor V-deficient plasma was from Biopool (Ventura, CA). Phospholipids were from Avanti Polar Lipids (Alabaster, AL), and vesicles composed of 80% phosphatidylcholine/20% phosphatidylserine (PCPS) were prepared by sonication. CPI, tissue-type plasminogen activator (tPA), and D-Phe-Pro-Arg chloromethylketone (PPACK) were from Calbiochem; uPA was a kind gift of J. Henkin (Abbott Laboratories); human prothrombin, alpha thrombin, factor Xa, and antithrombin were from Enzyme Research Laboratories (South Bend, IN); and TAFI, factor V, factor Va, and a monoclonal antibody against the human factor Va heavy chain were from Haematologic Technologies (Essex Junction, VT). Pentapharm Pefakit TAFI assay kit was from Centerchem (Norwalk, CT); chromozym t-PA substrate was from Roche Applied Science; S-2222, S-2765 and S-2238 substrates were from DiaPharma (West Chester, OH); Spectrozyme uPA substrate, and recombinant human factor VIIa were from American Diagnostica (Stamford, CT); heparin was from Fujisawa (Deerfield Park, IL); poly(L-lysine) hydrobromide, 3-(2-Furyl)acryloyl-Ala-Lys-OH (FAAK), salmon testis DNA, protamine sulfate, thrombin receptor agonist peptide (SFLLRN), and polyP were from Sigma. DNA was dissolved in 50 mM Tris (pH 7.5), 100 mM NaCl, 0.1% NaN_3, then sheared by repeated passage through a 23-gauge hypodermic needle until the viscosity was reduced. Recombinant human tissue factor was expressed in *E. coli* and purified as described (25, 26); recombinant human TFPI and blocking anti-TFPI antibodies were a kind gift of George Broze (Washington University, St. Louis); and recombinant human thrombomodulin was a kind gift of Timothy Mather (Oklahoma Medical Research Foundation, Oklahoma City).

Assays of Plasma Clotting and Fibrinolysis. Coagulometer-based clotting assays were performed at 37°C in a STart 4 coagulometer (Diagnostica Stago) using recombinant human tissue factor reconstituted into PCPS vesicles as described (27).

Microplate-based clotting assays were performed at 37°C in flat-bottom, polystyrene 96-well plates (Corning) by monitoring turbidity changes (A_{405}) using a VERSAmax microplate reader (Molecular Devices). Typical 100-μl reactions contained 30% human pooled normal plasma, 8 mM Tris pH 7.4, 0.008% Tween-20, and 10.6 mM calcium chloride in the presence or absence of polyP of various mean chain lengths. Combined clotting/fibrinolysis assays were also performed in microplates, except that 1.4 nM uPA was included in the reactions to activate fibrinolysis. In some combined clotting/fibrinolysis assays, rapid initiation of clotting was ensured by including 10 nM thrombin.

Preparation of Platelet Releasates. Platelets were obtained from the Central Illinois Regional Blood Bank and washed three times by centrifugation followed by gentle resuspension in platelet buffer (20 mM Hepes, pH 7.5/149 mM NaCl/5.55 mM D-glucose/2.68 mM KCl/1.05 mM $MgCl_2$/1.8 mM $CaCl_2$) at 6.0×10^9 platelets per ml. Platelets were stimulated with 20 μM thrombin receptor agonist peptide (SFLLRN) for 20 min at 37°C, then centrifuged at $3{,}000 \times g$ for 10 min. The supernatant (platelet releasate) was serially diluted in platelet buffer for clotting assays.

Factor V Activation. Rates of thrombin generation in mixed reactions were studied in a microplate format using the following final reactant concentrations: 0.33 nM factor Xa, 400 nM prothrombin, and either 6 nM factor V or 0.45 nM factor Va. The reactions also contained 17.5 mM Tris·HCl (pH 7.4), 80 mM NaCl, 8.3 mM $CaCl_2$, 16.7 μM PCPS, and 0.03% BSA, with or without 22.5 μM polyP of chain length 75 ($polyP_{75}$). Thrombin generation was detected by measuring the hydrolysis of the chromogenic thrombin substrate, S-2238, at 405 nm.

Factor V activation was also studied by mixing 100 nM factor V with either 1 nM factor Xa or 0.1 pM thrombin in 50 mM Tris·HCl (pH 7.5), 100 mM NaCl, 2 mM $CaCl_2$, 0.3% PEG-8000, and 20 μM PCPS, with or without 37.5 μM $polyP_{75}$. Reactions were incubated at 37°C; aliquots were removed at varying times and heated to 95°C for 5 min in SDS sample buffer. Samples were resolved on SDS/PAGE and transferred to poly(vinylidene difluoride) membranes, and the factor Va heavy chain was detected by using a monoclonal antibody (Haematologic Technologies). Antibody binding was detected by chemiluminescence (ECL) using a PhosphorImager.

Real-Time Monitoring of Thrombin Generation. Thrombin generation was quantified according to the method described by Hemker *et al.* (28) in a Fluoroscan Ascent fluorometer (Thermolabsystems, Helsinki, Finland) equipped with automated dispenser. Fluorescence was detected at an excitation wavelength of 390 nm and emission wavelength of 460 nm. Pooled normal plasma (80 μl) was dispensed into round-bottom 96-well plates (Immulon 2HB, Dynex), and the plate was warmed to 37°C for 5 min before automated addition of the starting reagent (20 μl per well), containing 0.5 pM relipidated tissue factor, 50 μM PCPS, 75 μM $polyP_{75}$, 2.5 mM fluorogenic substrate (Z-Gly-Gly-Arg-AMC·HCl), and 16.6 mM $CaCl_2$. Measurements were taken of four samples per min for 60 min, and data were analyzed by using the Thrombinoscope software package (Synapse Bv, Maastricht, Netherlands), which allows real-time quantitation of thrombin activity.

PolyP Digestion and Stability. $PolyP_{65}$ (100 μM) was digested for 2 h at room temperature with 100 units/ml calf intestinal alkaline phosphatase (Promega) in 50 mM Tris (pH 9.5), then immediately diluted for use in clotting assays. Digestion was confirmed by urea-PAGE and staining with toluidine blue. PolyP stability was measured by adding $polyP_{75}$ to human serum or heparinized plasma and quantifying polyP at time points after guanine isothiocyanate extraction as described (3).

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